Attorney Docket No. 25727

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Aviv SHAISH et al. Confirmation No. 1529

Serial No. 10/668,601 Art Unit: 1655

Filed: September 24, 2003 Examiner: FLOOD, M. C.

For: **THERAPEUTIC USES OF DUNALIELLA POWDER**

DECLARATION UNDER 37 C.F.R §1.131

The Commissioner for Patents
Washington, D.C. 20231

Sir:

NOW COME the undersigned and declare that:

1) I, Aviv SHAISH, a citizen of ISRAEL, have an address of Talmei Yehyel 200, 79810, ISRAEL.

2) I, Dror Harats, a citizen of ISRAEL, have an address of 71 Mendes Street, Ramat Ran, 52653, ISRAEL.

3) That we are the coinventors named in the above-identified application for Letters Patent.

4) The following facts show invention, i.e., at least conception, coupled with due diligence to a subsequent reduction to practice, of the presently claimed subject matter in ISRAEL, on a date prior to the Second day of July in the year TWO-THOUSAND AND THREE (07/02/2003), which date is, upon information and belief, the publication date (effective date) of the Hayashi reference entitled "METHOD FOR PRODUCING CAROTENOID," cited by the Examiner in the Official Action dated June 14, 2006. This date is hereinafter referred to as the "Hayashi publication date".

5) We invented the claimed subject matter of the present above identified application prior to the Hayashi publication date.

6) Attached hereto and made a part of this affidavit are Exhibits A through D.

7.1) Exhibit A is copy of the original Experimental Protocol, in Hebrew, taken from the laboratory notebook of Aviv SHAISH which corresponds to Example 7 set forth in the specification and was preformed under our direction. The experiment compared glucose levels observed in diabetic mice fed a standard diet and diabetic mice fed a diet including crude Dunaliella algae after diabetes induction. As can be deduced from Exhibit A, the experiment was commenced on May 5, 2003. The Experimental Protocol, Exhibit A, describes: separating the mice into a first group of mice (the control group) and a second group of mice (the experimental group); after three weeks inducing diabetes in the mice by administering STZ over a five day period; following another three weeks, administering Dunaliella to the experimental group of mice; and periodically testing the mice for blood plasma levels of glucose, insulin, cholesterol, and triglycerides.

7.2) From the Experimental Protocol, it can be seen the mice were first bled on May 5, 2003, at which time their blood glucose plasma levels were tested and recorded. *See* Exhibits C and D. The second bleeding occurred on May 29, 2003. STZ was administered to the mice over a five day period of beginning on June 1, 2003. Diabetes was thereby induced, as evidenced by the blood glucose plasma levels recorded on June 22, 2004. The third bleeding occurred on June 26, 2003. The mice were last bled on July 27, 2003.

8) Exhibit B is an English language translation of Exhibit A.

9) Exhibit C is a copy of a page out of the laboratory notebook of Aviv Shaish which contains data obtained from the experiment described in Exhibit A.

10) Exhibit D is an analysis of the data obtained and referenced in Exhibit C.

11) The work reported in each of Exhibits A through D was preformed in ISRAEL. The material written on the originals corresponding to these exhibits was written in ISRAEL. The invention was reduced to practice in ISRAEL.

12) We invented the presently subject matter, within the meaning of 37 CFR § 1.131, prior to the Second day of July in the year TWO-THOUSAND AND THREE (07/02/2003), which date is, upon information and belief, the publication date of the Hayashi reference.

13) All statements made herein of my own knowledge are true. All statements made herein upon information and are believed to be true. We understand that willful false statements and the like are punishable by fine or imprisonment, or both under the provisions of 18 U.S.C. § 1001, and may jeopardize the validity of the application or any patent issuing thereon.

14) Further, declarants sayeth naught.

WITNESS our signatures below on the indicated dates

25.9.2006
DATE

shaish aviv
Aviv SHAISH

25.9.2006
DATE

D. Harats
Dror Harats

EXHIBIT A

פרוטוקול ניסוי

עכברי -/- LDL P34 רקע מעורב זכרים מחולקים ל- 2 קבוצות ניסוי:

קבוצה 1 – טיפול: STZ
קבוצה 2 – טיפול: STZ + דונליאלה

בכל קבוצה 12/13 עכברים סה"כ 25 עכברים.
כל העכברים יואכלו בדיאטת WESTERN.

מהלך הניסוי:

PRETREATMENT במשך שלושה שבועות + שבועיים לאחר הזרקת STZ:
דיאטת CHAW לקבוצה 1 ודונליאלה+ CHAW לקבוצה 2.

1. דימום 1 – לפני התחלת ה – PRETREATMENT — תאריך: 5.5.03
2. חלוקה לקבוצות לפי תוצאות הפרופיל והתחלת ההאכלה. — תאריך: 8.5.03
3. דימום 2 – לאחר 3 שבועות — תאריך: 29.5.03
4. הזרקת STZ במשך 5 ימים. — תאריך: 1-5.6.03
5. לאחר שבועיים בדיקת גלוקוז לנוכחות סכרת — תאריך 22.6.03
6. דימום 3 – שלושה שבועות לאחר STZ – (האכלה western) — תאריך: 26.6.03
7. דימום 4 – לאחר ארבעה שבועות (western) — תאריך: 16.7.03
8. דימום 5 – לאחר ארבעה שבועות — תאריך: _____

בכל דימום יבדקו הפרמטרים הבאים:

- גלוקוז בצום
- אינסולין בצום
- כולסטרול וטריגליצרידים בצום

<u>לאחר 11 שבועות הקרבה</u>: לבבות לבדיקת טרשת וצביעות
פרופיל שומנים – כולסטרול וטריגליצרידים
מעיים, כבד
אינסולין, גלוקוז, A1C
FPLC
לפטין? TNFα?

<u>תומרים</u>:
STZ
סטיקים לבדיקת סוכר
קיט לאינסולין
דונליאלה
בופר ציטראט
WESTERN
כל קבוצה תחולק ל-2 כלובים סה"כ 4 כלובים.

EXHIBIT B

TRANSLATION OF PAGE FROM LABORATORY NOTEBOOK OF AVIV SHAISH

EXPERIMENTAL PROTOCOL

P34 LDL -/- mice mixed background males divided into 2 experimental groups:

Group 1 – treatment: STZ
Group 2 – treatment: STZ + Dunaliella

12/13 mice in each group total 25 mice
All of the mice will be fed a WESTERN diet

Course of the experiment

Pretreatment during three weeks + two weeks after STZ injection:
CHAW diet for Group 1 and CHAW + Dunaliella for Group 2.

1. Bleeding 1 – before beginning the pretreatment Date: __5.5.03_____
2. Division into groups according to the profile results and initiation of the feeding Date: __8.5.03_____
3. Bleeding 2 – after 3 weeks Date: __29.5.03_____
4. Injection STZ for a period of 5 days Date: __1-5.6.03_____
5. After 2 weeks measuring glucose to determine diabetes Date: __22.6.03_____
6. Bleeding 3 – 3 weeks after STZ – beginning feeding WESTERN/+Du Date: __26.6.03_____
7. Bleeding 4 – after 4 weeks Date: __16.7.03 – sacrificing
8. Bleeding 5 – after 4 weeks Date: _____

In each bleeding the following parameters will be checked:

- Fasting glucose
- Fasting insulin
- Fasting cholesterol and triglycerides

After 11 weeks sacrifice: Hearts for checking arteriosclerosis and coloring
Fats profile – cholesterol and triglycerides
Intestines, liver
Insulin, glucose, A1C
FPLC
Leptin? TNFα?

Materials:
STZ
Sticks for testing sugar
Insulin kit
Dunaliella
Citric buffer
WESTERN
Each group will be divided into 2 pages – total of 4 pages.

EXHIBIT C

glucose

STZ	5.5.03	29.5.03	15.6.03	22.6.03	9.7.03	16.7.03
6	89	96	410	308	432	409
7	113	98	188	285	392	599
8	113	118	366	389	511	600
12	78	86	73	344		
16	139	99	526	346	379	
18	82	38	85	246	593	
19	106	87	382	233	395	600
20	102	43	544	227		
21	120	64	308	263	346	
25	96	53	197	375	364	545
68	114	58	500	235	414	
74	121	60	121	280	378	514
	106.08	75.00	308.33	294.25	420.40	544.50

STZ (2) 9 STZ (3)

glucose

STZ+DU	5.5.03	29.5.03	15.6.03	26.6.03	9.7.03	16.7.03
39	100	132	301	425	301	565
40	92	62	178	291	395	546
41	133	116	337	269	240	600
42	84	109	306	369	349	600
43	63	71	445	337	227	230
45	143	99	366	364	368	366
47	74	97	435	366	346	288
49	103	110	393	354	462	600
50	118	66	82	380		
52	109	51	522	350	409	425
54	129	48	430	263	322	307
56	111	61	431	279	301	600
60	105	70	292	439	459	551
	104.92	84.00	347.54	345.08	348.25	473.17

EXHIBIT D

Thursday, July 24, 2003, 14:29:48

Data source: Data 1 in Notebook

Normality Test: Passed)P > 0.200(

Equal Variance Test: Passed)P = 0.662(

Group Name	N	Missing	Mean	Std Dev	SEM
stz glucose 9.7	12	2	420.400	75.905	24.003
dun glucose 9.7	13	1	348.250	75.709	21.855

72.150 Difference

(t = 2.223 with 20 degrees of freedom. (P = 0.038

percent confidence interval for difference of means: 4.451 to 139.849 95

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the .(input groups (P = 0.038

Power of performed test with alpha = 0.050: 0.462

.The power of the performed test (0.462) is below the desired power of 0.800
.You should interpret the negative findings cautiously

Plant Cell Physiol. 31(5): 689–696 (1990)
JSPP © 1990

Effect of Inhibitors on the Formation of Stereoisomers in the Biosynthesis of β-Carotene in *Dunaliella bardawil*

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An assay system was developed in which the effects of inhibitors of β-carotene biosynthesis in *Dunaliella bardawil* could be tested. Since *D. bardawil* can be induced to accumulate over 10% of its dry weight as β-carotene, it is particularly suitable for such studies. Norflurazon a desaturation inhibitor, caused the accumulation of phytoene, or of phytoene and phytofluene, depending on the concentration employed. J-334, a substituted 6-methylpyrimidine which also inhibits desaturation, caused the accumulation of β-zeacarotene, ζ-carotene and phytoene in different proportions, depending on the concentration employed. The cyclization inhibitors, nicotine, CPTA and MPTA, severely affected the growth and survival of the alga, and their effects could therefore not be studied directly. However, their action was observed indirectly by following the transformation of phytoene in norflurazon-pretreated phytoene-rich algae. Under these conditions, presence of the cyclase inhibitors caused the transformation of phytoene to lycopene, rather than to β-carotene. The accumulated β-carotene or the intermediates β-zeacarotene, lycopene, ζ-carotene, phytofluene and phytoene in *D. bardawil* were all composed of two stereoisomers, tentatively assigned as the all-trans stereoisomer (55%) and the 9-cis stereoisomer (45%). This suggests that the isomerization reaction which leads to the production of the presumed 9-cis isomers occurs early in the pathway of carotene biosynthesis, at or before phytoene, with no isomerization during the further transformations leading to β-carotene.

Key words: Carotene biosynthesis — Carotene inhibitors — *Dunaliella bardawil* — Herbicides.

Two strains of *Dunaliella*, *D. salina* Teod. and *D. bardawil*, produce large amounts of β-carotene under appropriate conditions (Ben-Amotz et al. 1982, Loeblich 1982). When *D. bardawil* is cultivated under high light intensity and growth limiting conditions, such as high sodium chloride concentration, nitrate deficiency or extreme temperatures, it accumulates β-carotene to more than 10% of the algal dry weight. Under non-accumulating conditions, it contains only about 0.3% (Ben-Amotz et al. 1982, Ben-Amotz and Avron 1983, 1989). The β-carotene is accumulated within oily globules in the interthylakoid spaces of the chloroplast and is composed mainly of two stereoisomers: 9-cis and all-trans (Ben-Amotz et al. 1982, 1988). The β-carotene content and the 9-cis to all-trans ratio are proportional to the integral light irradiance during a cell cycle (Ben-Amotz and Avron 1983, Ben-Amotz et al. 1987).

The biosynthesis of β-carotene (Fig. 1) can be divided into four stages: (a) formation of geranylgeranyl pyrophosphate from mevalonic acid; (b) condensation to form phytoene; (c) desaturation of phytoene to lycopene; and (d) cyclization of lycopene to form β-carotene. This pathway has been studied in higher plants, fungi and bacteria and to a lesser extent in algae (Goodwin 1980, Bauernfeind 1981). In *D. bardawil*, only one intermediate, phytoene, has been identified so far (Ben-Amotz et al. 1987). Algae treated with the herbicide norflurazon also accumulated two stereoisomers of phytoene in place of 9-cis and all-trans β-carotene. These data indicated that the isomerization reaction which eventually causes the accumulation of 9-cis and all-trans β-carotene occurs early in the pathway

Abbreviations: norflurazon, 4-chl-5-(methyl-amino)-2-(α,α,α-trifluoro-m-tolyl-3 (2H)-pyridazinine; CPTA, 2-(4-chlorophenyl) triethyl amine; MPTA, [2-(4-methylphenoxy)triethyl amine]; J-334, 6-(2-chlorobenzyloxy)-4-methyl-2-propylpyrimidine; 42-1668, 2,4-dichlorobenzoyl-4,4-dimethylcyclohexane-2,6-dione; 42-1669, 2,4-dichlorobenzoylcyclohexa-2,6-dione.

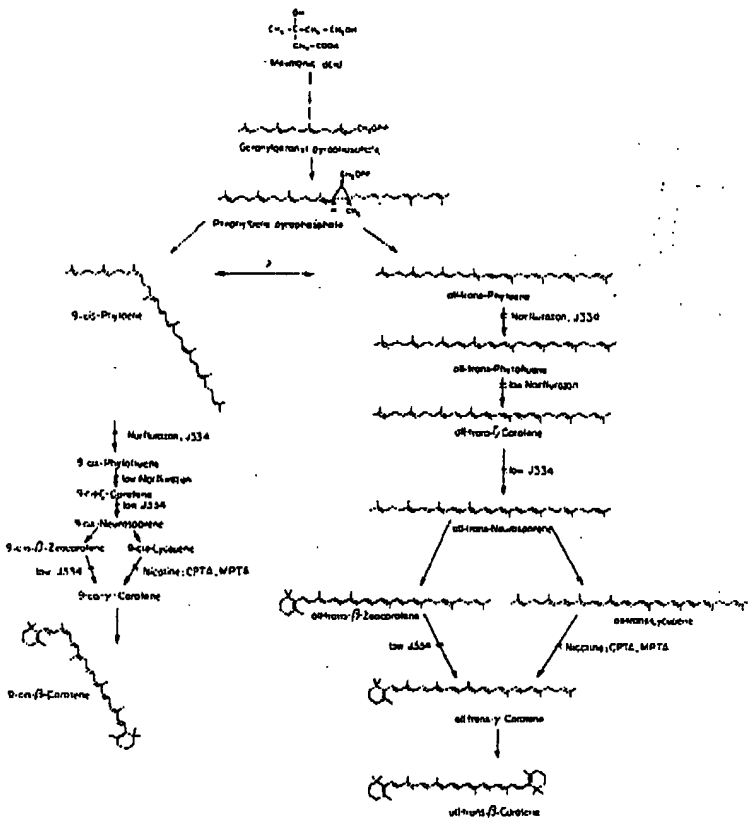

Fig. 1 Postulated pathway of all-trans and 9-cis β-carotene biosyntheis in *Dunaliella bardawil*. The concluded sites of inhibition of the inhibitors employed in this study are indicated.

of carotene biosynthesis (Ben-Amotz et al. 1987).

In this study we took advantage of the unique capability of *Dunaliella* to accumulate massive amounts of β-carotene mainly to answer the following questions: (a) Are the intermediates of the β-carotene biosynthetic pathway in *D. bardawil* similar to the intermediates in the pathway of other organisms? (b) Is the ratio between the two stereoisomers of the intermediates between phytoene and β-carotene similar to that of β-carotene under defined conditions? (c) Can *D. bardawil* be employed as a useful organism to analyze the mode of action of potential herbicides which act by affecting the carotene biosyntheic pathway?

Materials and Methods

Algae—Dunaliella bardawil Ben-Amotz & Avron, a local isolated species is deposited with the American Type Culture Collection, Rockville; MD, no. 30861.

Growth conditions—The algae were cultivated in a growth medium containing 1 M NaCl, 5 mM KNO_3, 5 mM $MgSO_4$, 0.2 mM $CaCl_2$, 0.2 mM KH_2PO_4, 2 μM $FeCl_3$, 5 μM Na_2EDTA, 7 μM $MnCl_2$, 1 μM $CuCl_2$, 1 μM $ZnCl_2$, 1 μM $CoCl_2$, 1 μM $(NH_4)_6Mo_7O_{24}$ and 50 mM $NaHCO_3$ (pH ~8.0). Algae were cultivated at 26–27°C with continuous light in a growth chamber illuminated with cool white fluorescent lamps of about 10 $W \cdot m^{-2}$. Lower light intensity was achieved by covering the flasks with Miracloth.

Growth parameters and pigments—Cell number was determined in a Coulter counter model Zm with a 100 μm orifice. For pigment analysis about 2 ml of algae were centrifuged at 1,000 × *g* for 10 min. The pellet was extracted by 3 ml of a solution of ethanol : hexane 2 : 1 (v/v), and centrifuged at 1,000 × *g* for 5 min. Following addition of 2 ml water and 4 ml of hexane, the hexane layer was separated and its spectrum determined. Samples for HPLC analysis were dried completely under N_2 and redissolved in a minimal volume of methylene chloride.

HPLC analysis—HPLC analysis was performed as described previously (Ben-Amotz and Avron 1989). For detection and quantitative analysis of the various pigments

the programmable multiwavelength detector was set at the following wavelengths and the following extinction coefficients ($E|^{1\%}_{1cm}$) were employed: lycopene, 504 nm (2,950); β-carotene, 450 nm (2,550); β-zeacarotene, 427 nm (1,940); ζ-carotene, 402 nm (2,550); phytofluene, 347 nm (1,540); and phytoene, 287 nm (915). The areas under each relevant peak with the appropriate extriction coefficient were used for quantitative estimations.

Inhibitors of β-carotene formation—Norflurazon = San 9789, Sandoz Co. Basel 13, Switzerland; Nicotine, Sigma Chemical Co., St. Louis, MO, U.S.A.; CPTA, Amchen Products Inc., Ambler, PA, U.S.A.; MPTA, Amchen Products Inc., Ambler, PA, U.S.A.; Imidazole, Sigma Chemical Co., St. Louis, MO, U.S.A.; J-334 = R11234, ICI Agrochemicals, U.K., kindly supplied by Dr. S. Ridley (Ridley 1982); 42-1668 and 42-1669, kindly supplied by Dr. P. F. Bocion and Dr. A. Binder of Zurich University.

Results

Developing an assay

The routine procedure to induce high β-carotene biosynthesis in *Dunaliella bardawil*, i.e. exposure to high intensity irradiation (Ben-Amotz and Avron 1983) can not be employed for examining the effects of inhibitors in the carotene biosynthetic pathway. It was previously shown (Ben-Amotz et al. 1987) that under such conditions *D. bardawil* loses its resistance to photobleaching resulting in rapid cell death (Ben-Amotz et al. 1989). We therefore adopted, as a standard assay for examining the effects of carotene biosynthesis inhibitors, a procedure in which β-carotene biosynthesis is induced at relatively low intensity (10 $W \cdot m^{-2}$) by starvation to nitrate (Ben-Amotz and Avron 1983).

Cells were pregrown with sufficient nitrate at very low intensity (5 $W \cdot m^{-2}$) in order to start with relatively low β-carotene containing cells. They were then washed twice and resuspended in a nitrate free medium and incubated under low intensity (10 $W \cdot m^{-2}$). Figure 2 illustrates the time course of β-carotene production under these conditions. Within 3–4 days the cells doubled to tripled their β-carotene content with no more than a doubling in the cell number. A much lower carotene accumulation, and a much larger cell proliferation, occurred when sufficient nitrate was also provided during the experimental period.

Desaturation inhibitors

Norflurazon—It was previously shown (Ben-Amotz et al. 1987) that the desaturation inhibitor, norflurazon, inhibits β-carotene production in *Dunaliella bardawil* while causing an equivalent accumulation of phytoene (see also Fig. 3, bottom, peak at 286 nm). When used at somewhat lower concentrations (Fig. 3, top) the accumulation of phytofluene, as indicated by the absorption at 330, 348 and

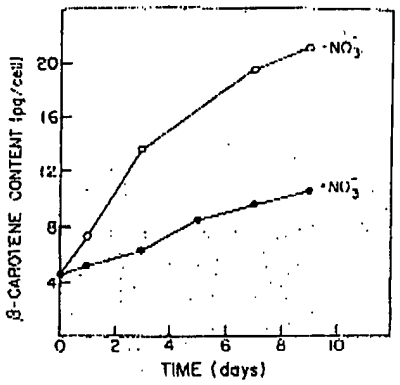

Fig. 2 Effect of nitrate deficiency on the β-carotene content of *Dunaliella bardawil*. Algae were grown in a medium containing 1 M NaCl under a low light intensity of 5 $W \cdot m^{-2}$, washed twice with nitrate-free medium and incubated in fresh medium at 3×10^5 cells $\cdot ml^{-1}$ under a light intensity of 10 $W \cdot m^{-2}$ with or without 5 mM nitrate, as indicated. Cell number at the end of the experiment was 5.5×10^5 cells $\cdot ml^{-1}$ in the nitrate free medium, and 1.7×10^6 in the nitrate sufficient medium.

366 nm, can also be detected. Thus, both the desaturation of phytoene to phytofluene, and that of phytofluene to ζ-carotene are differentially inhibited, depending on the con-

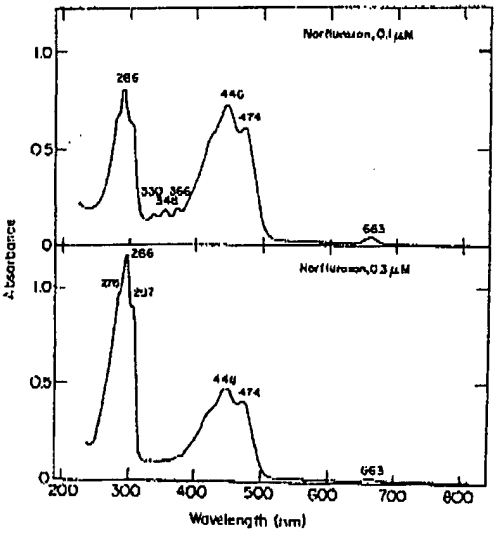

Fig. 3 Spectra of the pigments accumulated in norflurazon treated algae, showing phytoene and phytofluene. Experimental conditions as described in Figure 2. The inhibitor, at the indicated concentration, was added during transfer to the nitrate free medium. Pigments were extracted for analysis as indicated in Methods following 3 days of incubation.

centration of norflurazon employed.

J-334—The recently described carotenoid inhibitor J-334 (Ridley 1982), when used at high concentrations (4,000 nM) also inhibits fully β-carotene biosynthesis but promotes the accumulation of phytoene and ζ-carotene (Fig. 4, Table 1, peaks at 400, 426 nm). At lower concentrations, β-zeacarotene (peaks at 426, 452 nm), rather than phytoene was accumulated (Fig. 4, Table 1). Thus, depending on the concentration employed, different sites of desaturation were more prevalently affected resulting in the accumulation of different intermediates. The lower the concentrations, the intermediates preceding the further desaturation step are accumulated.

The identification of the indicated accumulated intermediates is more convincingly demonstrated by separation on HPLC and identification of the eluted peaks by on-line diode-array spectrophotometry. Figure 5 illustrates the separation of the pigments accumulated in the presence of 200 nM J-334. The elution was simultaneously monitored at the three indicated wavelengths and full spectra of the peaks of interest are presented in Figure 6. Although, as can be seen, some pigments overlap in their position during the HPLC separation, the combination of simultaneous monitoring at three wavelengths coupled with the on-line diode array spectrophotometry made it possible to unambiguously identify the accumulated intermediates.

As previously shown for β-carotene and phytoene in this alga (Ben-Amotz et al. 1987, 1988), also the other accumulated intermediates in the pathway of β-carotene biosynthesis occurred in two isomeric forms (in this case, β-zeacarotene and ζ-carotene). These are tentatively identified, in analogy with β-carotene (Ben-Amotz et al. 1988), as the all-trans and the 9-cis isomers. This will be discussed further below.

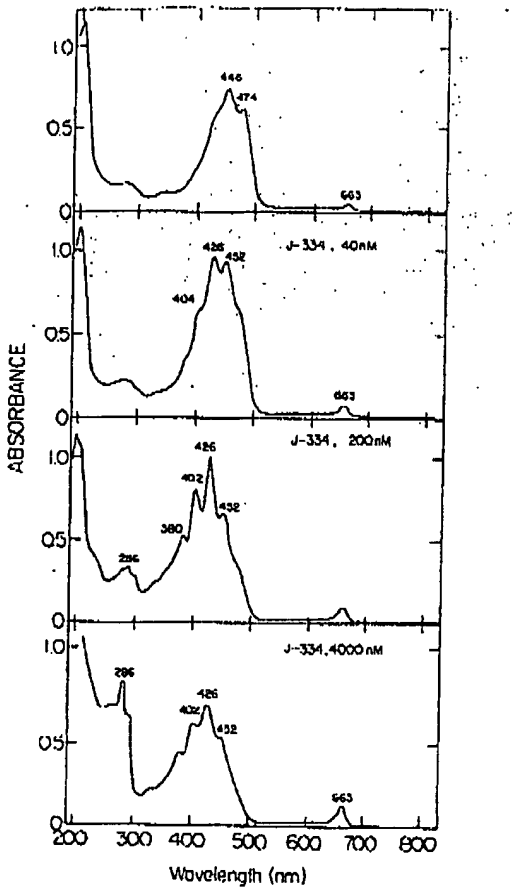

Fig. 4 Spectra of the pigments accumulated in J-334 treated algae. Conditions as described under Figure 3.

Cyclization inhibitors.

The cyclization inhibitors, Nicotine, CPTA and MPTA (Bramley and Mackenzie 1988, Britton 1979) when used directly severely inhibited growth and survival without accumulation of significant amounts of intermediates. How-

Table 1 The effect of J-334 concentration on pigment formation in *Dunaliella bardawil*

J-334, nM	β-carotene / chlorophyll g·g⁻¹ (%)	β-zeacarotene / Chlorophyll g·g⁻¹ (%)	ζ-carotene / chlorophyll g·g⁻¹ (%)	phytoene / chlorophyll g·g⁻¹ (%)
0.0	7.0 (98)	0.0 (0)	0 (0)	0.1 (2)
40	2.0 (27)	4.0 (55)	1.0 (14)	0.3 (4)
200	1.0 (13)	2.0 (27)	4.0 (53)	0.5 (7)
4,000	0.3 (4)	1.5 (19)	3.0 (38)	3.0 (39)

Conditions are as described in Fig. 2. J-334, at the indicated concentration was added during the transfer to the nitrate free medium. Pigments were extracted for analysis following 3 days of incubation. The column headed % indicate the relative proportion of the pigment in the total carotenoids analysed.

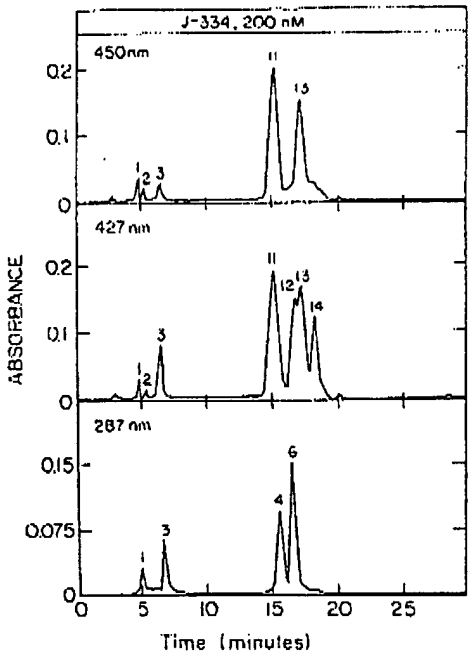

Fig. 5 HPLC analysis of the pigments accumulated by *Dunaliella bardawil* treated with 200 nM J-334. Experimental conditions as described under Fig. 3. Elution was simultaneously monitored at three wavelengths: 450, 427 and 287 nm. The peak numbers correspond to the following pigments: 1, lutein; 2, zeaxanthin; 3, chlorophyll *a*; 4, 9-cis phytoene; 6, all-trans phytoene; 11, all-trans β-zeacarotene; 12, all-trans ζ-carotene; 13, 9-cis β-zeacarotene; 14, 9-cis ζ-carotene.

ever, if the algae were preloaded with phytoene, by preincubation in the presence of the desaturation inhibitor, norflurazon (Fig. 3, bottom), the norflurazon removed and the cyclization inhibitors added, new intermediates were accumulated.

Figure 7 illustrates the results of such a treatment sequence with norflurazon and nicotine. The upper part of the figure illustrates the spectrum of an extract of untreated algae with the prominent accumulation of β-carotene. In the lower part, the accumulated phytoene (286 nm) is still evident, and in addition to β-carotene, nicotine induced the accumulation of lycopene (shoulder at 504 nm). Thus, nicotine inhibits the cyclization of lycopene to β-carotene. The extent of the inhibition can be monitored by the change in the ratio of absorption at 504 nm (lycopene) to that at 485 nm (β-carotene). As can be seen in Table 2, 100 μM nicotine induced an increase in that ratio from 0.19 to 0.69. Similar increases were induced by the cyclization inhibitors CPTA and MPTA. Imidazole, which presumably inhibits at an earlier step (see below) did not induce any change in the 504/485 nm ratio even though it markedly inhibits β-carotene biosynthesis (not shown).

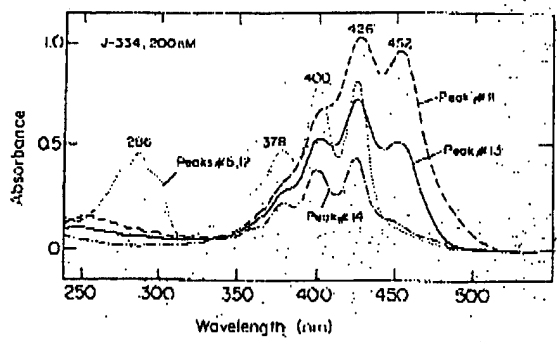

Fig. 6 Diode array on-line spectrophotometer absorption spectra of the major peaks indicated in Figure 5.

These conclusions are further substantiated by the separation of the accumulated pigments on HPLC and their identification in the on-line diode-array spectra. Figure 8, left, illustrates the pigments observed following treatment with norflurazon. Both 9-cis and all-trans phy-

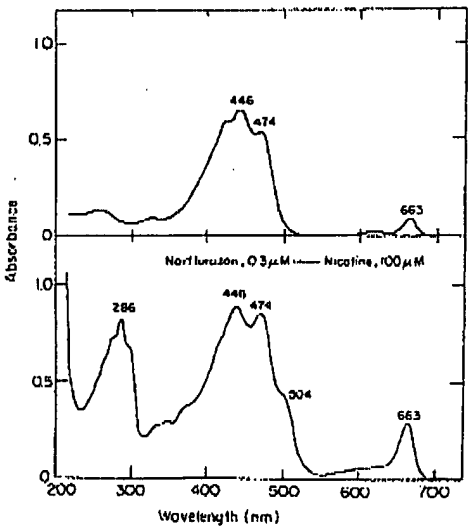

Fig. 7 Effect of nicotine on the conversion of phytoene to β-carotene and lycopene in *Dunaliella bardawil*. Conditions as described under Figure 2. Following growth for 3 days in the presence (lower half) or absense (upper half) of 0.3 μM norflurazon the phytoene-rich cells (see Fig. 3) were washed with nitrate-free medium and incubated for a further day in the same medium with (lower half) or without (upper half) 100 μM nicotine. Spectra of the pigments accumulated under the two treatments were taken as described under Methods.

Table 2 Effects of nicotine, CPTA, MPTA and imidazole on conversion of phytoene to β-carotene and lycopene

Treatment	Absorbance at 504 nm / Absorbance at 485 nm
Control	0.19
Nicotine, 100 μM	0.69
CPTA, 50 μM	0.62
MPTA, 50 μM	0.64
Imidazole, 1.0 mM	0.19

Conditions as described under Figure 7, except that nicotine was ommited or replaced by CPTA, MPTA, or imidazole at the indicated concentrations.

toene (287 nm) are clearly visible (see also Ben-Amotz et al. 1988). The center of the figure illustrates the pigments present following incubation of the norflurazon pretreated algae for one day in an inhibitor-free medium. Clearly the accumulated phytoene was converted to β-carotene (450 nm). The right side of the figure illustrates the pigments present following incubation of the norflurazon pretreated cells for one day in a fresh medium containing 100 μM nicotine. The accumulation of both 9-cis and all-trans lycopene (504 nm) is clearly evident. The identification of the pigments is made unambiguous by the simultaneously taken spectra of the peaks of interest, as illustrated in Figure 9.

Other inhibitors

Several of the inhibitors used inhibited β-carotene production, with no effect on growth, chlorophyll biosynthesis or survival, but did not induce the accumulation of products which can be detected spectrally. Among these were the new inhibitors 42-1668 and 42-1669 and imidazole (Ninet et al. 1969). Under the assay conditions used (Fig. 2), 10 μM of 42-1668, 10 μM of 42-1669 or 10 mM imidazole caused over 70% inhibition of β-carotene accumulation with no effect on the other parameters. It is possible that the site of inhibition of these compounds precedes prephytoene pyrophosphate, and therefore no visible intermediates can be observed in the detection procedures used in this study.

All-trans and 9-cis stereoisomers

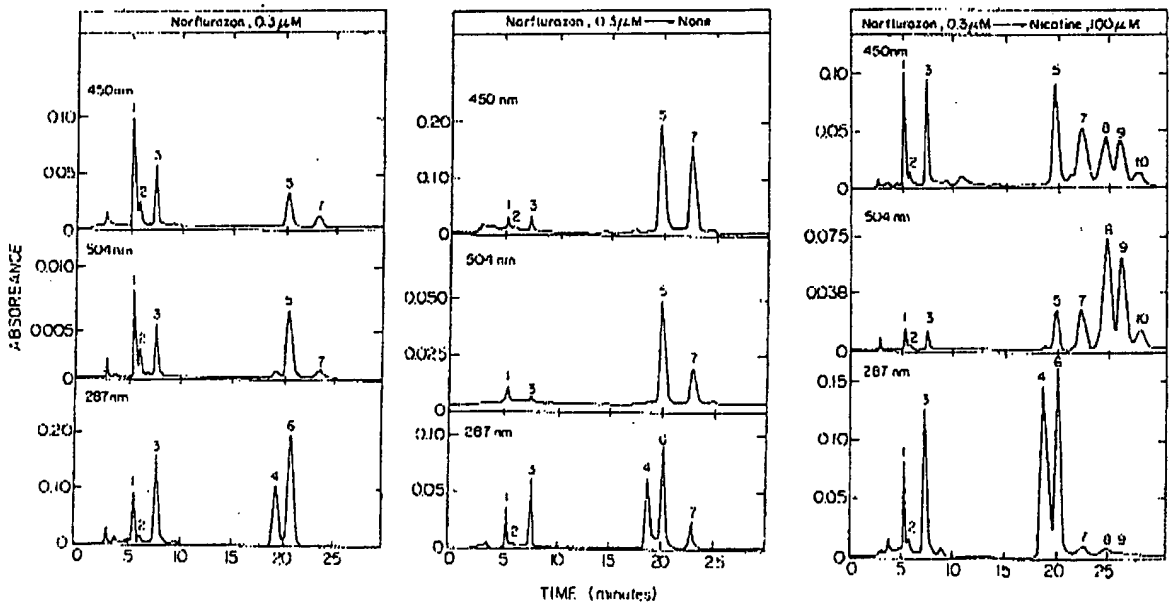

Fig. 8 HPLC analysis of the pigments accumulated by norflurazon pretreated *Dunaliella bardawil* incubated with nicotine. Experimental conditions were as described in Fig. 7. The left part illustrated separation of the pigments accumulated by the norflurazon treated algae. The central part, of these algae following a wash with a nitrate free medium and incubation in the latter medium for one extra day, and the right side when 100 μM nicotine was included in the medium of incubation for the extra day. The eluent was simultaneously read at 450 nm for β-carotene, 504 nm for lycopene and 287 for phytoene. Peak numbers are as indicated under Figure 5. New peaks were identified as: 5, all-trans β-carotene; 7, 9-cis β-carotene; 8, all-trans lycopene; 9, 9-cis lycopene; 10, unknown.

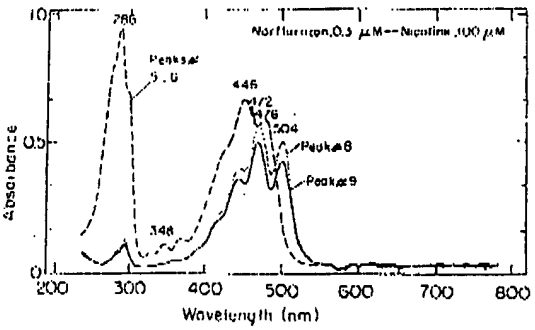

Fig. 9 Diode array on line spectrophotometer absorption spectra of the major peaks indicated in Figure 9 right.

In the presence of the different inhibitors, each of the β-carotene intermediates accumulated showed two major peaks when separated by HPLC. By analogy to β-carotene (Ben-Amotz et al. 1988), these were tentatively labelled as the all-trans and 9-cis stereoisomers. This was true for β-carotene, β-zeacarotene, lycopene, ζ-carotene, phytofluene and phytoene. Table 3 shows that the ratio between the two stereoisomers of each of the intermediates was around 0.8 under the conditions employed . These data support the suggestion (Ben-Amotz et al. 1988) that stereoisomerization occurs early in the biosynthetic pathway of β-carotene (at or before phytoene), with no further isomerization occurring during the following desaturation and cyclization steps (Fig. 1).

Discussion

The unique ability of *D. bardawil* to accumulate very large amounts of β-carotene under defined experimental conditions, makes it an excellent choice for studying the pathway of β-carotene biosynthesis. We developed techniques for utilizing this property of *D. bardawil* for analyzing the effect of a variety of inhibitors on the pathway of β-carotene biosynthesis. To avoid the destructive effect of high light intensity, usually employed to induce high carotene biosynthesis, we employed nitrate depletion at low intensities as the inductive process. Indeed, in contrast to studies with other organisms, addition of some of the studied inhibitors resulted in massive accumulation of the different biosynthetic intermediates of β-carotene, amounting to more than 3% of the algal dry weight. The high concentration of the accumulated intermediates eased the extraction, purification and identification of each intermediate in both of its stereoisomeric forms.

The desaturation inhibitors, norflurazon and J-334 inhibited predominantly different desaturation steps depending on the concentration employed. Thus, low norflurazon concentrations induced the accumulation of both phytofluene and phytoene, while at higher concentration only phytoene was accumulated (see Fig. 1).

The effect of J-334, a substituted 6-methyl pyrimidine, on the desaturation reactions was very striking. It almost completely inhibited β-carotene formation at submicromolar concentrations, while at the same time massive amounts of β-zeacarotene were accumulated. At slightly higher concentrations J-334 blocked the desaturation of ζ-carotene and the alga accumulated large amounts of ζ-carotene. Increasing the concentration of J-334 to the micromolar range caused inhibition of the phytoene desaturation reaction, and the accumulation of phytoene.

The cyclase inhibitors nicotine, CPTA and MPTA, inhibited the growth and survival of *Dunaliella bardawil* and therefore could not be used directly for studying their effect on the biosynthetic pathway of β-carotene. A two-step procedure was devised in which the cells were first incubated in the presence of norflurazon which caused the accumulation of phytoene, they were then washed free of norflurazon and treated with the cyclase inhibitors. With this procedure the inhibitors blocked the cyclization of lycopene, but allowed the transformation of phytoene to lycopene to proceed.

Table 3 Effect of different inhibitors on the ratio of the two stereoisomers in accumulated intermediates in the pathway of β-carotene biosynthesis in *D. bardawil*

Inhibitor	9-cis/all-trans				
	β-carotene	β-zeacarotene	lycopene	ζ-carotene	phytoene
None	0.78	ND	ND	ND	ND
Norflurazon, 300 nM	0.82	ND	ND	ND	0.84
J-334, 40 nM	0.80	0.78	ND	0.81	0.75
J-334, 200 nM	0.81	0.83	ND	0.85	0.78
Nicotine, 100 μM, + (norflurazon, 300 nM)	0.79	ND	0.84	ND	0.90

Conditions are as described under Figure 3–8. ND, not determined.

The two new inhibitors 42-1668 and 42-1669, and imidazole, most likely inhibit the biosynthesis of β-carotene between geranylgeranyl pyrophosphate and phytoene. They strongly inhibit β-carotene production, with no effect on chlorophyll content and with no accumulation of pigmented intermediates. Since geranylgeranyl pyrophosphate is also an intermediate in chlorophyll biosynthesis the site of inhibition is probably the one suggested above.

The presence of two stereoisomers of each of the identified β-carotene intermediates was noted with all the inhibitor studies. Previous evidence showed that light plays a major role in both the preponderance of 9-cis β-carotene and the increase in total β-carotene in *D. bardawil*. Both were shown to be proportional to the integral irradiance to which the culture is exposed during a division cycle (Ben-Amotz and Avron 1983, Ben-Amotz et al. 1988). Since all the intermediates between phytoene and β-carotene contained similar ratios of the two isomers the isomerization step must occur at or before phytoene, and no further isomerization is likely to occur during the further transformation of phytoene to β-carotene.

It is generally believed that lycopene is the precursor of cyclic carotenes (Britton 1979). However, the findings that lycopene synthesis but not β-carotene synthesis is inhibited when tomato ripens above 30°C or when it is treated by dimethyl sulphoxide (Goodwin and Jamikorn 1952, Raymund et al. 1967, Tomes et al. 1958) indicated that β-carotene can be produced also *via* cyclization of neurosporene to β-zeacarotene (see Fig. 1). We found that J-334 severely inhibited β-carotene formation while inducing large accumulation of β-zeacarotene and ζ-carotene but not lycopene. On the other hand, the cyclase inhibitors nicotine, CPTA or MPTA inhibited β-carotene production less severely but did induce moderate lycopene accumulation. These results suggest that both pathways of β-carotene biosynthesis are active in *D. bardawil* but that the quantitatively major one is that proceeding via β-zeacarotene.

The biochemical pathway drawn in Figure 1 summarizes the most likely pathway for the formation of the all-trans and 9-cis β-carotene stereoisomers in *D. bardawil*. It also illustrates the concluded sites of action of the various inhibitors employed in this study.

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